DRAFT: August 31, 1994

DECISION DOCUMENT
TSCA SECTION 5(H)(4) EXEMPTION FOR
ASPERGILLUS ORYZAE

I. SUMMARY

Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. It has also been used in the fermentation industry for production of enzymes and other organic compounds. There are conflicting opinions about whether A. oryzae can be isolated in nature. While it has been suggested that all strains of A. oryzae are natural variants of A. flavus modified through years of selection for fermenting foods, this theory has not been fully accepted in the scientific community. A. oryzae is regarded as having no pathogenicity for plants or animals, though there are a few reports of isolation of A. oryzae from patients. Products of A. oryzae fermentations seem to be associated with allergic responses in certain occupations with potentially high exposure to those materials, e.g., production of a-amylase. A. oryzae can produce a variety of mycotoxins when fermentation is extended beyond the usual time needed for these foods. Wild A. flavus isolated from the environment readily produce aflatoxins and other mycotoxins. A. oryzae, however, does not produce aflatoxin and has not been shown to be capable of aflatoxin production. The potential risks from use of A. oryzae in fermentation facilities are low.

II. BACKGROUND

A. Introduction

EPA recognizes that some microorganisms present a low risk when used under specific conditions at general commercial use. Therefore, EPA is proposing expedited regulatory processes for certain microorganisms under these specific conditions at the general commercial use stage. Microorganism uses that would be exempt meet criteria addressing: (1) performance based standards for minimizing the numbers of microorganisms emitted from the manufacturing facility; (2) the introduced genetic material; and (3) the recipient microorganism. Microorganisms that qualify for these exemptions, termed Tier I and Tier II, must meet a standard of no unreasonable risk in the exempted use.

To evaluate the potential for unreasonable risk to human health or the environment in developing these exemptions, EPA

focuses primarily on the characteristics of the recipient microorganisms. If the recipient is shown to have little or no potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is also specifying procedures for minimizing numbers of organisms emitted from the facility. When balanced against resource savings for society and expected product benefits, these exemptions will not present unreasonable risks.

B. Criteria for Minimizing Release from Manufacturing Facilities

The standards prescribed for the Tier I exemption require the following: (1) the structure(s) be designed and operated to contain the microorganism, (2) access to the structure should be limited to essential personnel, (3) inactivation procedures shown to be effective in reducing the number of viable microorganisms in liquid and solid wastes should be followed prior to disposal of the wastes, (4) features to reduce microbial concentrations in aerosols and exhaust gases released from the structure should be in place, and (5) general worker hygiene and protection practices should be followed.

1. Definition of structure. EPA considers the term "structure" to refer to the building or vessel which effectively surrounds and encloses the microorganism. Vessels may have a variety of forms, e.g., cubic, ovoid, cylindrical, or spherical, and may be the fermentation vessel proper or part of the downstream product separation and purification line. All would perform the function of enclosing the microorganism. In general, the material used in the construction of such structure(s) would be impermeable, resistant to corrosion and easy to clean/sterilize. Seams, joints, fittings, associated process piping, fasteners and other similar elements would be sealed.

2. Standards to minimize microbial release. EPA is proposing, for several reasons, a somewhat cautious approach in prescribing standards for minimizing the number of microorganisms emitted through the disposal of waste and the venting of gases. First, a wide range of behaviors can be displayed by microorganisms modified consistent with EPA's standards for the introduced genetic material. Second, EPA will not conduct any review whatsoever for Tier I exemptions. EPA believes the requirement to minimize emissions will provide a measure of risk reduction necessary for making a finding of no unreasonable risk. Taken together, EPA's standards ensure that the number of microorganisms emitted from the structure is minimized.

EPA's proposed standards for minimizing emission specify that liquid and solid waste containing the microorganisms be treated to give a validated decrease in viable microbial populations so that at least 99.9999 percent of the organisms resulting from the fermentation will be killed. Since the bacteria used in fermentation processes are usually debilitated, either intentionally or through acclimation to industrial fermentation, the small fraction of microorganisms remaining viable after inactivation treatments will likely have a reduced ability to survive during disposal or in the environment. Moreover, industrial companies, in an attempt to keep their proprietary microorganisms from competitors and to reduce the microbial numbers to those permitted by local sanitation authorities, modify the microorganisms to increase the ability of their microorganisms to survive and perform their assigned tasks in the fermentor but decrease their ability to survive in the environment external to the fermentor.

EPA requirements also address microorganisms in the exhaust from the fermentor and along the production line. To address exhaust from fermentors, EPA is proposing that the number of microorganisms in fermentor gases be reduced by at least two logs prior to the gases being exhausted from the fermentor. EPA selected this number based on an estimate of the numbers of microorganisms likely to be in the exhaust from an uncontrolled fermentor and common industry practice. Moreover, microorganisms that are physiologically acclimated to the growth conditions within the fermentor are likely to be compromised in their ability to survive aerosolization. EPA anticipates, therefore, that few microorganisms will survive the stresses of aerosolization associated with being exhausted in a gas from the fermentor. The provision requiring reduction of microorganisms in fermentor exhaust gases contributes to minimizing the number of viable microorganisms emitted from the facility.

EPA is also proposing that the requirements specify that other systems be in place to control dissemination of microorganisms by other routes. This would include programs to control pests such as insects or rats, since these might serve as vectors for carrying microorganisms out of the fermentation facilities.

3. Worker protection. The requirement to minimize microbial emissions, in conjunction with the requirement for general worker safety and hygiene procedures, also affords a measure of protection for workers. Potential effects on workers that exist with microorganisms in general (e.g., allergenicity) will be present with the microorganisms qualifying for this exemption. As with other substances that humans may react to

(e.g., pollen, chemicals, dust), the type and degree of allergenic response is determined by the biology of the exposed individual. It is unlikely that a microorganism modified in keeping with EPA's specifications for the introduced genetic material would induce a heightened response. The general worker hygiene procedures specified by EPA should protect most individuals from the allergenic responses associated with microorganisms exhausted from fermentors and/or other substances emitted along the production line. The EPA requirement that entry be limited to essential personnel also addresses this consideration by reducing to a minimum the number of individuals exposed.

4. Effect of containment criteria. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption. EPA is not specifying standards for minimizing the number of microorganisms emitted from the facility for microorganisms qualifying for Tier II exemption. Rather, the Agency requests that submitters utilize as guidance the standards set forth for Tier I procedures. The procedures proposed by the submitter in a Tier II exemption request will be reviewed by the Agency. EPA will have the opportunity to evaluate whether the procedures the submitter intends to implement for reducing the number of organisms emitted from the facility are appropriate for that microorganism.

C. Introduced Genetic Material Criteria

In order to qualify for either Tier I or Tier II exemption, any introduced genetic material must be limited in size, well characterized, free of certain nucleotide sequences, and poorly mobilizable.

1. Limited in size. Introduced genetic material must be limited in size to consist only of the following: (1) the structural gene(s) of interest; (2) the regulatory sequences permitting the expression of solely the gene(s) of interest; (3) the associated nucleotide sequences needed to move genetic material, including linkers, homopolymers, adaptors, transposons, insertion sequences, and restriction enzyme sites; (4) the nucleotide sequences needed for vector transfer; and (5) the nucleotide sequences needed for vector maintenance.

The limited in size criterion reduces risk by excluding the introduction into a recipient of extraneous and potentially uncharacterized genetic material. The requirement that the regulatory sequences permit the expression solely of the structural gene(s) of interest reduces risk by preventing

expression of genes downstream of the inserted genetic material. The limitation on the vector sequences that are components of the introduced genetic material prevents the introduction of novel traits beyond those associated with the gene(s) of interest. The overall result of the limited in size criterion is improved ability to predict the behavior of the resulting microorganism.

2. Well characterized. For introduced genetic material, well characterized means that the following have been determined: (1) the function of all of the products expressed from the structural gene(s); (2) the function of sequences that participate in the regulation of expression of the structural gene(s); and (3) the presence or absence of associated nucleotide sequences.

Well characterized includes knowledge of the function of the introduced sequences and the phenotypic expression associated with the introduced genetic material. Genetic material which has been examined at the restriction map or sequence level, but for which a function or phenotypic trait has not yet been ascribed, is not considered well characterized. Well characterized would include knowing whether multiple reading frames exist within the operon. This relates to whether more than one biological product might be encoded by a single sequence, and addresses the possibility that a modified microorganism could display unpredicted behavior should such multiple reading frames exist and their action not be anticipated.

3. Free of certain sequences. In addition to improving the ability to predict the behavior of the modified microorganism, the well characterized requirement ensures that segments encoding for either part or the whole of the toxins listed in the proposed regulatory text for the TSCA biotechnology rule would not inadvertently be introduced into the recipient microorganism.

These toxins are polypeptides of relatively high potency. Other types of toxins (e.g., modified amino acids, heterocyclic compounds, complex polysaccharides, glycoproteins, and peptides) are not listed for two reasons. First, their toxicity falls within the range of moderate to low. Second, these types of toxins generally arise from the activity of a number of genes in several metabolic pathways (multigenic).

In order for a microorganism to produce toxins of multigenic origin, a large number of different sequences would have to be introduced and appropriately expressed. It is unlikely that all of the genetic material necessary for metabolizing multigenic toxins would be inadvertently introduced into a recipient

microorganism when requirements that the genetic material be limited in size and well characterized are followed.

Similarly, other properties that might present risk concerns result from the interactive expression of a large number of genes. For example, pathogenic behavior is the result of a large number of genes being appropriately expressed. Because of the complex nature of behaviors such as pathogenicity, the probability is low that an insert consisting of well characterized, limited in size genetic material could transform the microorganisms proposed for exemption into microorganisms which display pathogenic behavior.

4. Poorly mobilizable. Poorly mobilizable means the ability of the introduced genetic material to be transferred and mobilized is inactivated, with a resulting frequency of transfer of less than 10^{-8} transfer events per recipient. The requirement that the introduced genetic material be poorly mobilizable reduces potential for transfer of introduced genetic sequences to other microorganisms in the environment. Such transfers would occur through the interaction of the introduced microorganism with indigenous microorganisms through conjugation, transduction, or transformation. Through such transfers, the introduced genetic material could be transferred to and propagated within different populations of microorganisms, including microorganisms which may never previously have been exposed to this genetic material. It is not possible to predict how the behavior of these potential recipient microorganisms will be affected after uptake and expression of the genetic material.

Since EPA is not limiting the type of organism that can serve as the source for the introduced genetic material, some limitation is placed on the ability of the introduced genetic material to be transferred. This limitation mitigates risk by significantly reducing the probability that the introduced genetic material would be transferred to and expressed by other microorganisms.

The 10^{-8} frequency is attainable given current techniques. Plasmids with transfer rates of 10^{-8} exist or are easily constructed. Some of the plasmids most commonly employed as vectors in genetic engineering (e.g., pBR325, pBR322) have mobilization/transfer frequencies of 10^{-8} or less.

The criteria set for "poorly mobilizable" for transduction and transformation should not prevent most microorganisms from meeting the exemption criteria, since the majority of transfer frequencies reported for transduction and natural transformation are less than 10^{-8}. Higher frequencies are likely only if the

introduced genetic material has been altered or selected to enhance frequency.

Fungal gene transfer has also been considered in development of the poorly mobilizable criterion. Although mobile genetic elements such as transposons, plasmids and double stranded RNA exist in fungi and can be readily transferred, this transfer usually is only possible between members of the same species during anastomosis, a process specific to fungi. Since anastomosis only occurs between members of the same species, the introduced genetic material would not be transferred to distantly related fungi as may occur with bacteria.

5. Effect of introduced genetic material criteria. The requirements placed on the introduced genetic material, in concert with the level of safety associated with Aspergillus oryzae, ensure that the resulting microorganisms present low or negligible risk. The probability is low that the insertion of genetic material meeting EPA's criteria into strains of A. oryzae will change their behavior so that they would acquire the potential for causing adverse effects. Risks would be mitigated by the four criteria placed on the introduced genetic material, the relative safety of A. oryzae, and the inactivation criteria specified for the Tier I exemption. In the case of Tier II exemption, risks would be mitigated in light of the four criteria placed on introduced genetic material, the relative safety of A. oryzae, and EPA's review of the conditions selected.

D. Recipient Microorganism Criteria

Six criteria were used by EPA to determine eligibility of recipient microorganisms for the tiered exemption. Microorganisms which EPA finds meet these criteria are listed as eligible recipients. The first criteria would require that it be possible to clearly identify and classify the microorganism. Available genotypic and phenotypic information should allow the microorganism to be assigned without confusion to an existing taxon which is easily recognized. Second, information should be available to evaluate the relationship of the microorganism to any other closely related microorganisms which have a potential for adverse effects on human health or the environment. Third, there should be a history of commercial use for the microorganism. Fourth, the commercial uses should indicate that the microorganism products might be subject to TSCA jurisdiction. Fifth, studies are available which indicate the potential for the microorganism to cause adverse effects on human health and the environment. Sixth, studies are available which indicate the survival characteristics of the microorganism in the environment.

After each microorganism was reviewed using the six evaluation criteria, a decision was made as to whether to place the microorganism on the list. The Agency's specific determination for Aspergillus oryzae is discussed in the next unit.

III. EVALUATION OF ASPERGILLUS ORYZAE

A. History of Use

1. History of safe commercial use. A. oryzae is used in the production of many different oriental foods such as soy sauce, sake and miso. As a "koji" mold, A. oryzae has been used safely in the food industry for several hundred years. It is also used to produce livestock probiotic feed supplements. The koji mold enzymes were among the first to be isolated and commercialized nearly 100 years ago. A. oryzae is considered a Class 1 Containment Agent under the NIH Guidelines for Research Involving Recombinant DNA Molecules. In Europe, Aspergillus species are considered category 2 under the European Federation of Biotechnology guidelines and category 1 under the OECD containment scale.

2. Products subject to TSCA jurisdiction. A. oryzae is currently used in the production of organic compounds such as glutamic acid and several enzymes that are of potential use commercially, for example, amylase, protease and b-galactosidase. While these enzymes could be used as TSCA products, they are more often used in food processing. In 1989, EPA reviewed a premanufacture notice (PMN) for a strain of A. oryzae modified for enhanced production of a lipase enzyme to be used primarily in detergent formulations for the removal of fat-containing stains. In 1994, EPA reviewed a PMN for a similar strain of A. oryzae modified for enhanced production of a cellulase gene for use in detergents as a color brightening agent.

B. Identification of Microorganism

1. Classification. The genus Aspergillus represents a grouping of a very large number of asexual fungi whose taxonomy is based on morphological features. The genus has been divided into groups based on attributes of the spores, conidiophores, and sclerotia. Because this separation of individual species into groups is based on morphological or physiological characteristics, it has resulted in somewhat tenuous and overlapping classification. While it has been hypothesized that A. oryzae is a domesticated version of A. flavus, a species known to produce potent aflatoxins, this theory has not been fully

accepted by the scientific community. While A. oryzae and A. flavus are closely related, the industrially used A. oryzae strains can be distinguished from A. flavus.

2. Related species of concern. A. oryzae is a member of the A. flavus group of Aspergillus species. Most of the members of this group are known to produce potent mycotoxins, including aflatoxins. Chromosomal DNA homology and other techniques have shown strains of A. oryzae and A. flavus to be essentially indistinguishable. It has been assumed that A. oryzae is a domesticated version of A. flavus that has been selected for use in foods because of its low probability of mycotoxin production.

C. Risk Summary

1. Studies regarding potential for adverse effects. A. oryzae strains can produce a variety of mycotoxins after extended fermentation; however, only a few strains are known to produce the more potent toxins. There have been reports of occupational asthma associated with use of A. oryzae; allergenicity appears to be associated with the a-amylase produced by the fungus. A. oryzae does not appear to be a significant human pathogen, nor has it been reported as a plant or animal pathogen. Although A. oryzae strains appear stable under cultivation, in theory there remains a remote probability that reversion to A. flavus phenotype could occur, if rearrangement of genetic material rather than deletion is the mechanism by which the A. flavus phenotype is lost. A. oryzae is not known to produce aflatoxins.

2. Studies regarding survival in the environment. There are conflicting opinions about whether A. oryzae can be isolated from the environment. A. oryzae seems to be a species created by domestication of A. flavus wild type and therefore may have lost certain features important to survival in the environment. Although soil is a possible natural habitat for A. oryzae, the intrinsic features of domesticated strains are expected to limit their ability to survive in a natural environment.

IV. BENEFITS SUMMARY

Substantial benefits are associated with this proposed exemption. Aspergillus oryzae is already widely employed in general commercial uses, some of which are subject to TSCA reporting. The Agency believes this exemption will result in resource savings both to EPA and industry without compromising

the level of risk management afforded by the full 90 day review. In addition to assessing the risk of A. oryzae, EPA has developed criteria limiting the potential for transfer of and expression of toxin sequences, and the conditions of use specified in the exemption are met (Tier I) or will be reviewed by EPA to ensure adequate risk reduction (Tier II). EPA requirements for minimizing numbers of viable microorganisms emitted are within standard operating procedures for the industry, and both the procedures and the structures specified in the exemption are the type industry uses to protect their products from contamination.

The exemption will result in reduced reporting costs and a decrease in delay associated with reporting requirements. The savings in Agency resources can be directed to reviewing activities and microorganisms which present greater uncertainty. This exemption should also facilitate development and manufacturing of new products and the accumulation of useful information.

V. RECOMMENDATION AND RATIONALE

A. RECOMMENDATION: Aspergillus oryzae is recommended for section 5(h)(4) tiered exemption.

B. RATIONALE

1. Risks from use of the recipient microorganism A. oryzae are low. A. oryzae has a history of commercial use without reports of adverse effects to workers or the environment. While some strains of A. oryzae are known to produce mycotoxins, these mycotoxins are not highly toxic to humans and their production under usual commercial conditions does not appear to pose a significant risk to human health. There is incomplete evidence to substantiate the theory that A. oryzae is a domesticated derivative of A. flavus, which is known to produce aflatoxins. However, aflatoxin production does not appear to be a problem for established A. oryzae strains under usual fermentation conditions. Attention to these fermentation conditions contribute to controlling the amount and timing of exposure to mycotoxins in the industrial setting. Furthermore, the use of proper safety precautions, good laboratory practices, and proper protective clothing, allays concern for exposure of workers to mycotoxins potentially produced by this microorganism. A. oryzae appears to lack many survival features necessary for establishment in the environment. Potential hazards to the public and the environment are also mitigated by limitations to exposure brought about by the conditions of contained use which

are designed to limit release of the microorganisms to the environment.

2. Use of strains of A. oryzae which are eligible for the TSCA section 5(h)(4) exemption present no unreasonable risk. Industrial strains of A. oryzae are not known to produce aflatoxins; however, it is possible that because A. oryzae appears to be a domesticated version of A. flavus it may possess dormant genes for aflatoxin production. As part of proving eligibility for this TSCA section 5(h)(4) exemption, companies are required to certify that they are using A. oryzae. It is therefore expected that companies will have information in their files which documents that their strains are A. oryzae and are not aflatoxin producers. Additionally, it is expected that companies will choose well-characterized industrial strains for further development through genetic modification. These expectations in combination with the use of Good Laboratory Practices should ensure the use of the correct species.

The limitations placed by the section 5(h)(4) exemption on the introduced genetic material, in particular the well-characterized and limited in size restrictions, should reduce the likelihood that any sequences relating to aflatoxin production could be introduced. The containment requirements would limit exposure to any mycotoxins produced.

Because the recipient microorganism was found to have little potential for adverse effects, introduced genetic material meeting the specified criteria would not likely significantly increase potential for adverse effects. As further assurance that risks would be low, EPA is specifying procedures for minimizing numbers of organisms emitted from the facility for the Tier I exemption and will be reviewing the conditions selected for the Tier II exemption. When balanced against resource savings for society and expected product benefits, this exemption will not present unreasonable risks.

REQUEST FOR COMMENTS

The Risk Assessment requests that comment be sought on the following: (1) The conclusion that genetic modification of A. oryzae cannot inadvertently produce an aflatoxigenic strain, and (2) whether there is a need to differentiate between strains of A. oryzae having long histories of safe use and strains similar in phenotype which are more recent isolates.

Attachment 1:

INTEGRATED RISK ASSESSMENT OF

Aspergillus oryzae

I. INTRODUCTION

Aspergillus oryzae is an asexual, ascomycetous fungus used for hundreds of years in the production of soy sauce, miso and sake without recorded incidents. There are conflicting opinions about whether *A. oryzae* can be isolated in nature. Although the details of the genetic relationship between *A. oryzae* and *A. flavus* remain unclear, the two species are so closely related that all strains of *A. oryzae* are regarded by some as natural variants of *A. flavus* modified through years of selection for fermenting of foods. *A. oryzae* is regarded as not being pathogenic for plants or animals, though there are a handful of reports of isolation of *A. oryzae* from patients. Products of *A. oryzae* fermentations, e.g. a-amylase, seem to be associated with allergic responses in certain occupations with high exposure to those materials. *A. oryzae* can produce a variety of mycotoxins when fermentation is extended beyond the usual time needed for these foods. While wild *A. flavus* isolates readily produce aflatoxins and other mycotoxins, *A. oryzae* has not been shown to be capable of aflatoxin production.

History of Commercial Use and Products Subject to TSCA Jurisdiction

Aspergillus oryzae has apparently been an essential part of oriental food production for centuries and is now used in the production of many different oriental foods such as soy sauce, sake and miso. Potential uses under TSCA include fermentations of numerous enzymes, e. g., amylase, protease and B-galactosidase, and organic compounds such as glutamic acid. While these products have a variety of potential commercial uses, they are mostly frequently used in food processing.

The experience of safe commercial use of *A. oryzae* is extraordinarily well established. As a "koji" mold it has been used safely in the food industry for several hundred years. *A. oryzae* is also used to produce livestock probiotic feed supplements. Even the commercialization of byproducts of the fermentation was established nearly a century ago. The "koji" mold enzymes were among the first to be isolated and commercialized. In 1894, Dr. J. Takamine isolated and sold

Takadiastase from a commercial firm he started in Clifton, New Jersey (Bennett, 1985a).

EPA has reviewed, under TSCA, two genetically modified strains of *A. oryzae* used for the production of enzymes (P89-134 and P94-1475).

II. IDENTIFICATION AND TAXONOMY

A. Overview

The candidate species is a member of the genus *Aspergillus* and belongs to the group of fungi that are generally considered to reproduce asexually (Fungi Imperfecti or Deuteromycetes), although perfect forms (forms that reproduce sexually) of some aspergilli have been found. The form genus *Aspergillus* represents a taxonomic grouping of a very large number of asexual fungi which are characterized by the production of spores on large black or brown conidia in phialides arranged on a characteristic spherical conidiophore termed the vesicle. This definition leads to inclusion of a complex assortment of organisms within the taxon. To simplify the taxonomy of such a large number of organisms, the genus *Aspergillus* has been divided into sections or groups based on color, size and roughness of the spore, conidiophore and vesicle as well as the arrangement of phialides and the presence of sclerotia. The separation of individual species into groups is somewhat tenuous and based on distinguishing measured characters with overlapping means. This resulted in the 132 species arranged in 18 groups by Raper and Fennell (1965) due to overlapping morphological or physiological characteristics. However, it is important to remember that taxonomy is "dealing with living variable organisms and that species and group concepts must be reasonably elastic" (Raper & Fennell, 1965).

As is the case of many fungi, the taxonomy of *Aspergillus* is primarily based on morphological features, rather than physiological, biochemical features and genetic characteristics often used to classify bacteria. Nomenclature problems of the genus *Aspergillus* arise from their pleomorphic life cycle. The newer findings show that this group of fungi has both a perfect (teleomorphic) and an imperfect (anamorphic) state.

The morphological approach to taxonomy has led to the existence of several synonyms for the genus *Aspergillus*. They are: *Alliospora* Pim; *Aspergillonsis* Spegazzini; *Cladaspergillus* Ritg; *Cladosparum* Yuill and Yuill; *Euaspergilus* Ludwig; *Gutturomyces* Rivolta; *Raperia* Subramaniam and Grove; *Sceptromyces* Corda; *Spermatoloncha* Spegazzini; *Sphaeromyces* Montagne;

Sterigmatocystis Cramer; and *Stilbothamnium* Hennings (Bennett, 1985b).

Aspergilli are ubiquitous in nature. They are geographically widely distributed and have been observed in a broad range of habitats, because they can colonize a wide variety of substrates.

B. The *Aspergillus flavus* Group

Aspergillus oryzae is a member of the *A. flavus* group of *Aspergillus* species. The *A. flavus* group, which also now includes *A. sojae, A. nomius* and *A. parasiticus* (see below) is defined by the production of spore chains in radiating heads which range in color from yellow-green to olive brown. The conidiophores are roughened and colorless. The spores themselves have conspicuous ridges and echinulations (spines). Sclerotia are occasionally produced (Raper & Fennell, 1965). *A. oryzae/flavus* species have never been connected to a sexual or teleomorphic stage. However, the teleomorphic stages of other *Aspergillus* species have been demonstrated by the formation of cleistothecia. These species belong to the genera *Emericella, Neosartorya* and *Eurotium,* all belonging to the ascomycetous family *Eurotiaceae* (Fennel, 1973). Either the sexual stages of the *A. flavus* group have not been recognized as such, being identified as completely different species based on morphology, or this group of fungi are "degenerate", having lost the ability to form sexual spores and mycelia.

A. oryzae is considered by some experts to be a domesticated variant of *A. flavus* (Kurtzman et al. 1986). Through long-time use, *A. oryzae* strains seem to have been selected to exhibit reduced sporulation, have more aerial mycelia and exhibit no environmental survival structures like sclerotia or the presence of aflatoxins that might function to inhibit grazing by insects. These morphological features that differentiate *A. oryzae* from *A. flavus* may represent adaptations to the artificial culture conditions of the koji fermentation. Misidentification of new isolates not obtained from well established cultures is always a possibility, since the key morphological differences between the two species seem related to culture adaptation. However, the source of *A. oryzae* strains for industrial fermentations today is likely to be standard culture collections. Environmental isolates of aspergilli would likely be identified as *A. flavus* rather than the laboratory-adapted *A. oryzae*.

C. Related Species of Concern

The taxonomy of *Aspergillus* has public health implications due to the production of potent mycotoxins by members of this genus. Most notable is the association of aflatoxins with members of the *A. flavus* group (Bennett, 1985b; Semeniuk et al., 1971). *A. oryzae* is a member of that group and in spite of the above mentioned morphological distinctions, *A. oryzae* appears to be very closely related to *A. flavus*. Numerous studies have been done to distinguish the koji molds from their toxicogenic relatives. The results are unambiguous in their confirmation of the conspecificity of *A. oryzae* and *A. flavus.* (see Section IV. below).

In a similar way, A. *sojae* is considered to be a domesticated form of *A. parasiticus* and shares a 92% DNA homology with its wild progenitor. *A. sojae* also has a history of safe use in the food industry. *A. parasiticus* in nature is an active colonizer of cereal grains and seeds with concurrent mycotoxin production. While these species can be distinguished from *A. flavus/oryzae* using morphological criteria, all four species intergrade. The hazard concerns for these species, thus, are equivalent to those associated with *A. flavus/oryzae.*

A. nomius is a newly classified species of toxigenic strains originally described in the *A. flavus* group, but not having the same level of DNA homology as shown among the four varieties mentioned above (Kurtzman et al., 1987). *A. nomius* produces aflatoxin and includes strains isolated from diseased bees. *A. oryzae* is distinguishable both morphologically and genetically from *A. nomius*.

III. HAZARD ASSESSMENT

A. Human Health Hazards

1. Toxin Production by *A. oryzae*

The close relationship between *A. oryzae* and *A. flavus* and the production of highly toxic mycotoxins by the latter has resulted in careful examination of the toxigenic potential of *A. oryzae*. However, *A. oryzae*, as a koji mold, has significant toxigenic potential in its own right. Those aspergilli used for manufacture of Japanese fermented foods have long been called koji molds. Prominent among the 25 koji molds listed is *A. oryzae* (Manabe et al., 1984). This fungus is used for Sake, an

alcoholic beverage, Miso, a soy bean paste, Shoyu, soy sauce, Amasake, a sweet beverage and Shouchu, a distilled liquor.

A. flavus commonly colonizes damaged cereal grains, soybeans and peanuts, actively producing mycotoxins (L. Stoloff, 1982). Certain strains of *A. oryzae* have themselves been shown to produce the mycotoxins aspergillic, kojic, cyclopiazonic and B-nitropropionic acids and maltoryzine (Ciegler & Vesonder, 1987).

Even with the food industry strains, a caveat of safety is that the fungal incubation not exceed the normal three day period. *A. oryzae* has been shown to produce toxic compounds under incubations longer than the typical koji fermentation (Semeniuk et al, 1971; Yokotsuka & Sasaki, 1986). The following are toxins produced by some strains of *A. oryzae*.

a. Kojic acid

Kojic acid (discovered by Saito, 1907) is produced by koji, a solid culture of the koji mold. It is a commonly produced metabolite that possesses antibacterial and antifungal activity. Few oral studies exist for this byproduct. Giroir reported toxic effects on chickens at four to eight mg/kg feed. Older studies (Friedemann, 1934, Werch et al. 1957, Morton et al., 1945) using intravenous or intraperitoneal challenges show moderate toxicity for kojic acid. Later work had similar results (Ueno and Ueno, 1978). Kojic acid also is reported to have moderate cardiotoxic and cardiotonic activity (Manabe et al., 1984., Bajpai et al. 1982). Nineteen of 47 *A. oryzae* strains tested produced kojic acid (Manabe et al., 1984). Even though it is apparent that the koji molds, including *A. oryzae* can produce the toxin kojic acid, this toxin may not be present in the fermented foods. The incubation period for sake, shoyu and miso is about two days and no kojic acid is found at that time (Manabe et al., 1984). However, these authors concluded that they were unable to prove kojic acid was not present in any fermented food in Japan, because conditions of production and materials were different for each industry, and were often uncontrolled. Semeniuk et al. (1971) warned that even with food industry strains, fungal incubation must not exceed three days. Thus, as the culture adjusts to changing conditions, *A. oryzae* may produce toxic compounds when incubation time exceeds typical koji fermentation time.

b. Maltoryzine

Maltoryzine, another toxic metabolite isolated and characterized by Iisuka and Iida (1962), was produced by *A. oryzae* var. *microsporus*. This metabolite was determined to be

the cause of poisoning among dairy cows. While highly toxic (LD_{50} 3 mg/kg; Iizuka, 1974; Ciegler and Vesonder, 1987), the substance may only be found in one or a very few strains of *A. oryzae*. The single isolate, IAM 2950, produced enough of the toxin when grown on malt rootlets to poison some milk cows, prompting the determination of its LD_{50}. The production of these toxins is related to the composition of the growth substrate and usually occurs in stationary phase cultures. Commercial strains of *A. oryzae* and *A. sojae* apparently do not produce maltoryzine.

c. Cyclopiazonic acid

Pitt and Cruickshank (1990), note that many isolates of *Aspergillus oryzae* are found to produce cyclopiazonic acid. Orth (1977), reporting on food industry strains of *A. oryzae*, indicated that eight of 16 strains produced cyclopiazonic acid. This acid is a natural contaminant of foods and feeds and is produced by several molds including those used in fermented food production. These included *A. flavus, A. versicolor, A. tamarii*, several *Penicillium* species, including *P. camemberti*, and *A. oryzae.* This mycotoxin has been shown to occur naturally in corn, cheese, peanuts and in Kodo millet that was implicated in natural human intoxication in India (CAST Task Force Report No. 116, 1989a). Benkhemmar et al. (1985) showed that when cyclopiazonic acid producing (CPA+) strains are mated with CPA- strains, the CPA+ phenotype is dominant in the heterokaryon. Oral administration produced effects at levels ranging from 0.25 to >50 mg/kg with dogs among the most sensitive species and rats among the least (Purchase, 1971; Nuehring et al., 1985). LO(A)ELs for sensitive species were at or under 1mg/kg. Nishie et al. (1985) noted that Rao and Husain (1985) identified cyclopiazonic acid as the cause of debilitating illnesses in cattle and man in India.

d. b-nitropropionic acid

A. oryzae can produce b-nitropropionic acid, along with other food-borne molds (Gilbert et al., 1977). Its mode of action is apparently irreversible succinate dehydrogenase inhibition which can cause a variety of symptoms often neurological in nature. These symptoms have been studied in mice (Gould and Gustine, 1982; Umezawa, 1967) and rats (Hamilton and Gould, 1987) where intravenous or subcutaneous LD_{50}s of 20-50 mg/kg were determined. Reports of livestock poisoning via ingestion in feed (James et al., 1980; James, 1983) showed that ingestion of b-nitropropionic acid could produce significant toxic effects up to and including death. When *A. oryzae* (ATCC 12892) was studied for its ability to produce b-nitropropionic acid on various high protein and carbohydrate-rich foods, it

flourished and produced this toxin in cooked sweet potato, potato and ripe banana (Penel and Kosikowski, 1990). Ames type assays for mutagenicity (Dunkel, 1985) showed positive responses with and without activation for two *Salmonella* strains, but not for three others. This assay uses multiple indicator strains in order to ensure that each potential mutation mode is detectable; the failure in three strains merely implies that the mutation modes to which each is sensitive are not the ones associated with the test substance.

2. Taxonomic and Genetic Relationship to Other Aspergilli

The closest taxon to *A. oryzae* is *A. flavus* which Kurtzman et al. (1986) regard as conspecific. Many strains of *A. flavus* produce aflatoxins which are acutely toxic to mammals (oral LD_{50}s ranging from 1 to 15 mg/kg depending on test species (Ceigler, 1975). Aflatoxins are animal carcinogens (Barnes and Butler, 1964; Dickens and Jones, 1964; Sinhuber, 1968) and also probable human carcinogens (Council for Agricultural Science and Technology, 1989). Developmental effects have also been found (Elis and DiPaolo, 1967, Le Breton et al., 1964).

While the koji molds like *A. oryzae* are distinguishable from, they are nevertheless very closely related to, *A. flavus*. Distinguishing between *A. oryzae* and *A. flavus* by physical traits is elusive. The toxicogenic subspecies/variety *A. flavus* has numerous spores chains that remain yellow-green; sterigmata that are always biseriate; spiny (echinulate) individual spores; roughened conidiophores up to 600µm in length and sclerotia often present. The variety called *A. oryzae* specifically has fewer spore chains, fading to brown with age; longer average conidiophores (about two to three mm); smoother individual spores; sterigmata usually in 1 series and sclerotia rarely produced (Raper & Fennel, 1965).

3. Lack of Aflatoxin Production in *A. oryzae*

Despite this strong similarity between the two species, production of aflatoxins has not been demonstrated by *A. oryzae.* Many studies affirm that the currently available strains confirmed to be *A. oryzae* are not capable of producing aflatoxins (Wei and Jong, 1986; Yokotsuka and Sasaki, 1986). In one test, no strains of *A. oryzae* or *A. sojae* (another koji mold) produced detectable levels of aflatoxins, while 33% and 85% of the strains of *A. flavus* and *A. parasiticus*, respectively, were toxigenic. As mentioned above, Kurtzman, et al. (1986) regard *A. oryzae* and *A. sojae* as domesticated varieties of their respective subspecies. Only one study (El-Hag and Morse, 1976) describes

aflatoxin production by a strain reported to be *Aspergillus oryzae* (NRRL strain 1988). This observation is notable as an exception to the rule of no aflatoxin production by *A. oryzae.*

It has been noted that *A. flavus* strains upon extended laboratory cultivation lose morphologically distinguishing characteristics, making them appear much like *A. oryzae* (Kurtzman, et al., 1986). Wicklow (1984) details the competitive disadvantages of *A. oryzae* and implies that *A. flavus* is the "wild" form. Kurtzman, et al., (1986) ask whether the separation between toxigenic and non-toxigenic *A. flavus* group species occurs through ecological adaptation or chromosomal changes such as translocations or inversions.

The elucidation of metabolic pathways responsible for the production of aflatoxins by *A. flavus* group fungi has progressed rapidly. Recently Payne (Bhatnagar, et al. 1992 and Payne, 1994) reported on the conversion of an aflatoxin non-producing strain of *A. flavus* to aflatoxin B_1 positive using a cosmid library developed from a toxigenic *A. flavus*. While added metabolic precursors could not stimulate toxin production in the mutant, the addition of an appropriate cosmid carrying a <5 Kbp fragment of the genome of the toxin producer converted the non-toxigenic strain to significant levels of aflatoxin production. Further work has resulted in isolation of a small segment specifying a regulatory, rather than structural, gene that affects early parts of the pathway. Probes for this regulatory gene, designated *afl* R, have been positive in both *A. oryzae* and *A. sojae*, even though those strains do not produce aflatoxin. In addition, Payne stated that probes for structural genes for aflatoxin production were also positive in some, but not all, *A. oryzae* strains examined.

It appears that evidence is mounting towards multiple reasons for failure to produce aflatoxins in *A. oryzae* cultures. One explanation is a lack of functional regulators, specifically *afl* R, that activate aflatoxin production. Another is that some or all of the structural genes in the aflatoxin pathway may be non-functional. For both types of genes, those sequences could be absent or present in the wrong orientation or split by insertions or modified slightly so as to be non-functional. Except for substantial deletion or absence of the necessary sequences, all of these alternatives are potentially reversible. However, Payne indicated that he doubted that industrial strains of *A. oryzae* were likely to revert to aflatoxin production. He indicated that, even though probes found the presence of appropriate gene sequences, the genes so detected could easily be incomplete enough so as to be completely non-functional.

Thus, complete absence of genetic potential is not the only plausible explanation for the non-expression of characters such as aflatoxin production in *A. oryzae*. In a related study, researchers attempting to improve strains of a mold identified as *A. oryzae* used for food fermentation in Thailand acquired a toxin producing strain by simple UV mutagenesis of a known "safe" strain (Kalayanamitr, et al. 1987). The toxins produced by this strain and other toxicogenic *A. oryzae* strains are not aflatoxins but rather other types of mycotoxins. The exact composition of the toxins involved in *A. oryzae* toxicosis in these studies, as in other anecdotal studies, was not determined (Semeniuk, et al., 1971; Wicklow and Dowd, 1989, and Kalayanamitr, et al., 1987). The mechanism for this conversion to toxigenicity was not investigated, but the mutations required could have affected either structural or regulatory genes and produced the new observed toxigenic phenotype.

4. Colonization and Pathogenicity

Aspergillus oryzae does not appear to be a significant human pathogen. Available information documents infections in humans possibly caused by *A. oryzae* in only three instances. The first was a case of meningitis (Gordon, et al., 1976). In the second case, *A. oryzae* invaded the paranasal sinuses, causing fever and right periorbital swelling (Byard, et al., 1986). The third case was a pulmonary aspergilloma caused by *A. oryzae* (Liao, 1988). Care must be exercised in evaluating these three cases as having been caused by this organism due to its close taxonomical relationship to *A. flavus* and the possibility of incorrect identification. The relative rarity of such cases in light of the commonplace use of *A. oryzae* suggests this species has a low potential for expressing pathogenic traits.

5. Allergic Reactions to *Aspergillus oryzae*

Allergic reactions are not uncommon for aspergilli and many occupational cases have been described for *A. oryzae*. Baker's asthma is reported to be the most frequent occupational lung disease in Switzerland and West Germany (Wuthrich and Baur, 1990). The a-amylase, produced by *A. oryzae*, that is used by bakers in bread making, was reported by Birnbaum, et al. (1988) to have caused asthma in a baker. Based on an observation of a case of baker's asthma due to monovalent sensitization to a-amylase used as an additive to flour, investigators tested 31 bakers who had occupational asthma and/or rhinitis by skin tests and serologic RAST examinations. Thirty-two percent of the bakers had RAST specific IgE to a-amylase from *A. oryzae.* Akiyama, et al. (1987) describe a case of allergic bronchopulmonary aspergillosis due to *Aspergillus oryzae* in a 19-

year-old female.

6. Conclusions

There are two major concerns for human health hazards associated with *A. oryzae*. The first, most directly tied to *A. oryzae*, is for mycotoxin production after extended fermentation. A variety of toxins can be produced, with the most common being the moderately toxic kojic acid. Other more potent toxins may only be produced by a few strains or in lesser quantities. The second issue is that of the nearly indistinguishable identity of *A. oryzae* and *A. flavus*. The identification issue raises questions as to the likelihood that strains identified as the latter produce highly toxic aflatoxins and the anecdotal evidence that *A. oryzae* produces no aflatoxins.

The first concern is mitigated by the limited toxicity of the most commonplace toxin and by the fact that toxin production is minimized through use of appropriate fermentation controls.

The second concern is more complex. Anecdotal evidence gathered over centuries suggests that *A. oryzae* commercial food strains do not produce aflatoxins nor have there been reports of any human healths effects from aflatoxin. However, *A. oryzae* appears so closely related to its aflatoxin-producing counterpart as to be viewed as consisting of culture-attenuated strains of *A. flavus* (Kurtzman, 1994; Wicklow, 1984). It has been hypothesized that *A. oryzae* evolves under culture from *A. flavus* strains due to selection for features that would be ecologically detrimental in the wild. Wicklow (1984) details the competitive disadvantages of *A. oryzae* and implies that *A. flavus* is the "wild" form. This is similarly suggested for the *A. sojae* - *A. parasiticus* pairing. Kurtzman, et al. (1986) have shown that *A. flavus* and *A. oryzae* are essentially the same based on DNA comparisons. It is reasonable to conclude that under years of growth in laboratory culture, *A. oryzae* can no longer express genes for toxin production.

Hypothetically, then, if *A. oryzae* has evolved to non-aflatoxigenic status after centuries in culture, the question remains whether it can revert to the "wild" type. The experience of oriental food production would seem to suggest not, or at least not frequently enough as to be detectable. Recent studies (Payne, 1994; Klich, 1994) suggest homology between parts of the *A. oryzae* genome and structural genes for aflatoxin production. It is conceivable that reintroduction of regulatory genes or their gene products could activate a dormant aflatoxin synthetic potential. There is no evidence to show that the required gene transfer or gene rearrangement that might provide the needed

functional sequences for an aflatoxin producing *A. oryzae* strain occurs naturally. The question is, therefore, whether this type of genetic modification is possible in culture.

B. Environmental Hazards

1. Hazards to Animals

The potential for toxin production is the main environmental hazard issue of concern for *A. oryzae.* If there were a method to distinguish between toxicogenic and non-toxicogenic strains, there would be no environmental concern for *A. oryzae.* Two recent studies that addressed the question of differentiating between toxin producing and non-toxicogenic strains of the related species *A. flavus*, *A. parasiticus* and *A. nomius* were unable to correlate either mitochondrial or chromosomal DNA RFLPs with mycotoxin production (Moody & Tyler, 1990a, 1990b). This again points to differences that may only involve small regulatory regions or that involve differences in structural gene complements that are beyond the detection limit of current DNA typing technologies.

Compounding this is the observation that *A. oryzae* and *A. flavus* are essentially indistinguishable by most molecular techniques. *A. flavus* is believed to be second in frequency only to the frank fungal pathogen, *A. fumigatus*, as a cause of aspergillosis in many species. *A. flavus* is associated specifically with invasive diseases of insects as well as toxicosis (Austwick, 1965). Recently, some insect pathogenic *A. flavus* strains were reclassified into *A. nomius* (Kurtzman et al., 1987). Whether *A. oryzae* is involved depends on how one defines the species of the *A. flavus* group. The effects on livestock of the various toxins that occur after extended koji fermentations, or in contaminated feed, show that the "minor" mycotoxins can still cause economic loss. No anecdotal accounts have been found that demonstrate that these potential effects occur in wildlife outside the agricultural environment.

2. Hazards to Plants

No reports of *A. oryzae* effects on living plants have been found. This species does not appear to be pose a hazard to plants.

3. Conclusions

The issues for environmental hazards are similar to those for human health hazards. The primary hazard concerns are for toxin production by *A. oryzae* strains. Under usual conditions of

culture, well established commercial strains of this species do not seem to produce significant levels of mycotoxins, although certain moderately potent toxins can be produced after extended culture. Aflatoxins appear not to be produced by such cultures. The potential for environmental hazard is dependent on the likelihood that commercial strains could escape and establish themselves in the wild and grow under conditions analogous to those resulting in toxin production in extended culture. The few examples of livestock poisoning associated with the "minor" toxins, b-nitropropionic acid, maltoryzine and cyclopiazonic acid cited above imply that, for a short time at least, strains of *A. oryzae* may be able to survive in the wild.

IV. EXPOSURE ASSESSMENT

A. Worker Exposure

Aspergillus oryzae is considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules (U.S. Department of Health and Human Services, 1986). In Europe, *Aspergillus* spp. are treated as low-risk-class microorganisms, i.e., category 2 of the European Federation of Biotechnology (Frommer et al., 1989) or category 1 on the OECD containment scale. Category 1 of the European Federation of Biotechnology scale includes organisms deemed harmless, which can be grown under good industrial large scale practices (GILSP), while category 2 organisms like *Aspergillus* require more stringent containment.

No data were available for assessing the release and survival specifically for fermentation facilities using *A. oryzae*. Therefore, the potential worker exposures and routine releases to the environment from large-scale, conventional fermentation processes were estimated on information available from eight premanufacture notices submitted to EPA under TSCA Section 5 and from published information collected from non-engineered microorganisms (Reilly, 1991). These values are based on reasonable worst-case scenarios and typical ranges or values are given for comparison.

During fermentation processes, worker exposure is possible during laboratory pipetting, inoculation, sampling, harvesting, extraction, processing and decontamination procedures. A typical site employs less than 10 workers/shift and operates 24 hours/day throughout the year. NIOSH has conducted walk-through surveys of several fermentation facilities in the enzyme industry and monitored for microbial air contamination. These particular facilities were not using recombinant microorganisms, but the

processes were considered typical of fermentation process technology. Area samples were taken in locations where the potential for worker exposure was considered to be potentially greatest, ie. near the fermentor, the seed fermentor, sampling ports, and separation processes (either filter press or rotary drum filter). The workers with the highest potential average exposures at the three facilities visited were those involved in air sampling. Area samples near the sampling port revealed average airborne concentrations ranging from 350 to 648 cfu/m^3. Typically, the Chemical Engineering Branch would not use area monitoring data to estimate occupational exposure levels since the correlation between area concentrations and worker exposure is highly uncertain. Personal sampling data are not available at the present time. Thus, area sampling data have been the only means of assessing exposures for previous PMN biotechnology submissions. Assuming that 20 samples per day are drawn and that each sample takes up to 5 minutes to collect, the duration of exposure for a single worker will be about 1.5 hours/day. Assuming that the concentration of microorganisms in the worker's breathing zone is equivalent to the levels found in the area sampling, the worst-case daily inhalation exposure is estimated to range up to 650 to 1200 cfu/day. The uncertainty associated with this estimated exposure value is not known (Reilly, 1991).

B. Environmental and General Exposure

1. Fate of Organism

Environmental exposure to *A. oryzae* is significantly affected by the ability of strains to survive outside controlled fermentation conditions. However, *A. oryzae* is seen by some factions in the fungal taxonomy community to be a species created by domestication of *A. flavus*. This process of domestication has apparently resulted in the development of traits that would limit survival, and thus, the exposure potential of *A. oryzae*. One character that is correlated with the survival of the wild type *A. flavus* organism is also a specific environmental hazard concern: mycotoxin production. It appears that mycotoxin production and other wild type *A. flavus* characters are fungal responses to insect predation pressures and long term survival and dispersal needs (Wicklow, 1983; Wicklow and Dowd, 1989; Wicklow and Shotwell, 1983). The spiny nature of the spores, the roughened character of the conidiophore and the production of toxic metabolites such as mycotoxins are hypothesized to deter insects from eating the fungal mycelium and spores (Wicklow, 1983). As with many other advantageous characters, there is an assumed metabolic cost associated with the production of these mycelial features and secondary metabolites to prevent insect predation.

Typical wild type *A. flavus* strains isolated from soil or molded grains will, upon continual subculturing, develop longer conidiophores and produce fewer spores, sclerotia and more aerial mycelium (Wicklow, 1983). Several strains originally described as *A. flavus* when isolated were renamed *A. oryzae* when reexamined after years of maintenance by subculturing (Wicklow, 1983). These morphological changes probably resulted from selection pressures similar to those imposed upon industrial "koji" strains and reaffirms the fact that cultural maintenance can itself substantially alter strain character, if the organism is not in a quiescent state.

With release from insect predation and survival insured by human manipulation, the wild type fungus could express some otherwise non-adaptable characters. These include characters associated with the "koji" molds: abundant aerial hyphae, less sporulation and larger spores, lack of sclerotia and mycotoxin production. Like etiolated seedlings, the lanky conidiophores and aerial mycelia of *A. oryzae* would not be resilient to environmental perturbations and probably are not advantageous for natural substrate utilization. Aerial mycelia would also interfere with spore dispersal and larger spores themselves would disperse less well than the wild type found in *A. flavus.* Larger spores may aid in faster initial colonization of the substrate in the industrial strains (Wicklow, 1984). Both *A. oryzae* and *A. sojae* have been shown to have spores that germinate about three hours sooner than their possible environmental progenitors *A. flavus* and *A. parasiticus* (Wicklow, 1984).

The following sections review the potential exposure of populations and the environment outside of a fermentation facility to an industrially used strain of *A. oryzae*. Despite the possible limitations of survival of *A. oryzae* relative to *A. flavus*, a conservative approach is taken. Because no data are available regarding the ability of industrial strains to disperse and persist in the environment, this assessment is based on mathematical models. These models assume that the microorganisms are dispersed as if they were particles, and that they neither multiply nor die during the dispersal process. These are reasonable assumptions for spores, but they are less appropriate for vegetative forms.

2. Releases

Estimates of the number of *A. oryzae* organisms released per production batch are tabulated in Table 1. The minimally controlled scenario assumes no treatment of the fermentor off-gas and assumes 100-fold (2 log) reduction of the maximum cell density of the fermentation broth resulting from inactivation

(Reilly, 1991). The containment criteria required for the full exemption scenario assume the use of in-line filters to treat vent gases and a 99% removal efficiency under normal operating conditions. They also assume an overall 6-log reduction relative to the maximum cell density of the fermentation broth resulting from inactivation steps (Reilly, 1991).

TABLE 1. Estimated Number of Viable Aspergillus oryzae Organisms Per Production Batch

Release Media	Minimally Controlled (cfu/day)	Full Exemption (cfu/day)	Release (days/year)
Air Vents	$2x10^{8}$ - $1x10^{11}$	$2x10^{6}$ - $1x10^{9}$	350
Rotary Drum Filter	250	250	350
Surface Water	$7x10^{12}$	$7x10^{8}$	90
Soil/Landfill	$7x10^{14}$	$7x10^{10}$	90

Source: Reilly, 1991

3. Air

There are no specific data regarding the survivability of *A. oryzae* in the atmosphere after release. Human exposure to *A. oryzae* aerosols, should it occur, would occur via inhalation. Releases from fermentor off-gas may result in nonoccupational inhalation exposures, if the releases are outside of the fermentation facility. To estimate these potential exposures, Versar (1991) used the sector averaging form of the Gaussian algorithm described in Turner (1970) and the release rates estimated by Reilly (1991) and summarized in Table 1. For purposes of this assessment, a release height of three meters and downward contact at a distance of 100 meters were assumed. Under the minimally controlled scenario of no removal of organisms by treatment of off-gasses, ambient human inhalation exposures are estimated to range from 9×10^{6} to 4×10^{9} cfu/day. For systems operating in compliance with criteria required for the full exemption, and thus with 99% reduction of the off-gasses, exposures of 9×10^{4} to 4×10^{7} cfu/day are estimated.

According to Versar (1991), these estimates represent hypothetical exposures under reasonable worst case conditions. However, it should be noted that these exposures are actually substantial overestimates, because industrial fermenters, which may have volumes of 100,000 liters (Finkelstein et al., 1989), themselves are higher than three meters, the assumed stack height [the taller the stack, the lower the predicted organism

concentration at 100 meters downwind]. In addition, one of the conditions of the exemption is that the fermentors are not vented directly to the ambient air.

4. Water

Versar (1991) estimated the concentrations of *A. oryzae* in surface water using stream flow values for water bodies receiving process wastewater discharges from facilities within SIC Code 283 (drugs, medicinal chemicals, and pharmaceuticals). The surface water release data (cfu/day) tabulated in Table 1 were divided by the stream flow values to yield a surface water concentration of the organism (cfu/L). The stream flow values for SIC Code 283 were based on discharger location data retrieved from the Industrial Facilities Dischargers database on December 5, 1991, and surface water flow data retrieved from the RXGAGE database. Flow values were obtained for water bodies receiving wastewater discharges from 154 indirect (facilities that send their waste to a POTW) and direct dischargers (facilities that have an NPDES permit to discharge to surface water). Tenth percentile values indicate flows for smaller rivers within this distribution of 154 receiving water flows and 50th percentile values indicate flows for more average rivers. The flow value expressed as 7Q10 is the lowest flow observed over seven consecutive days during a 10-year observation period. The use of this methodology to estimate concentrations of *A. oryzae* in surface water assumes that all of the discharged organisms survive wastewater treatment and that growth is not enhanced by any component of the treatment process.

Estimated concentrations of *A. oryzae* in surface water for minimally controlled and full exemption scenarios are tabulated in Table 2.

TABLE 2. Aspergillus oryzae Concentrations in Surface Water

Flow	Receiving Stream Flow (MLD*)		Organisms (cfu/l)	
	Mean	Q710	Mean	Q710
Minimally Controlled				
10th Percentile	156	5.60	4.5×10^{4}	1.25×10^{6}
50th Percentile	768	68.13	9.11×10^{3}	1.03×10^{5}
Full Exemption				
10th Percentile	156	5.60	4.5×10^{0}	1.25×10^{2}
50th Percentile	768	68.13	9.11×10^{-1}	1.03×10^{1}

*MLD = million liters per day
Source: Versar, 1991

5. Soil

Because soil is a possible natural habitat for *A. oryzae*, long-term survival in soil is expected. Human exposures via dermal contact and ingestion may occur at the solid waste disposal site, if the strain can establish itself. Environmental exposures to terrestrial, avian, and aquatic (via runoff) organisms may also take place. However, it should be noted that for establishment to take place, the introduced *A. oryzae* will probably have to out-compete and displace the indigenous *A. flavus* and *A. oryzae* populations. The limitations cited above may make this difficult.

6. Summary

Although, in order to produce a quantitative estimate of exposure for this species, a conservative estimate is the only possible approach given available data, the intrinsic features of the domesticated strains of *A. oryzae* may limit exposure beyond the calculated values. *A. oryzae* may not exist in the wild, but it may exist in the immediate surroundings of fermentation plants. The issue of exposure to a strain of this species is basically a question of whether the increase in release of spores, due to production of these strains, will be noticeable when compared to normal exposure to other production strains. In that context, the number of spores of strains released from production facilities, under the conditions described in Table 2, would not appear to add significantly to the environmental burden

of spores produced from other production sources. Added to this is the limited probability that new production strains would fare any better than similar established production strains in the ability to survive in a natural environment. Given these conditions, concern for environmental exposure to this species would be limited to the immediate vicinity of the production plant.

V. INTEGRATION OF RISK

In the previous sections, information regarding the potential exposures and hazards to workers, the general public, animals, plants and the environment was reviewed. This section serves to integrate this information to evaluate the potential risks associated with the industrial use of *Aspergillus oryzae*.

A. Discussion

The only major concerns identified are associated with human and animal toxicity due to mycotoxin production. *A. oryzae* and *A. flavus* are designations of taxa that represent the extremes of a spectrum of traits associated with a common fungus. Current evidence points to *A. oryzae* as a domesticated derivative of *A. flavus*. The evidence is not complete enough to indicate whether *A. oryzae* represents a unique genotype as well as a stable phenotype. It appears that under prolonged cultivation the phenotype of *A. oryzae* will be exhibited and that aflatoxins will not be produced from such strains. Other toxins such as cyclopiazonic acid and kojic acid may, however, be expressed.

1. Aflatoxin Production

Although it is likely that *A. oryzae* held in cultivation for decades or even centuries are likely to represent strains having small, but key, deletions in an otherwise identical genome to *A. flavus*, it is remotely possible that the phenotypic differences between the two species may be due to differences in the arrangement and control of genes rather than the loss or gain of them. If *A. oryzae* strains have had reversible gene modifications that prevent the expression of aflatoxin genes, then environmental control of such rearrangements is possible and reversion can occur. It must be noted that there have been no reports of workers in the industrial setting suffering from aflatoxin effects.

Additionally mitigating this concern is the observation that *A. oryzae* introduced to the environment in industrial fermentation wastes is not likely to survive for any extended

period of time due to its loss of key survival features. Wicklow (1984) has described the competitive disadvantages of *A. oryzae.* These observations suggest that this organism is highly adapted to conditions in the laboratory.

There is a basic question as to the likelihood that *A. oryzae* exists in the wild. Some researchers (Klich, 1994) indicate that *A. oryzae* can be isolated in nature. If these reports cannot be dismissed as artifacts of detection methodology, or mis-designation of the strains, they indicate that either *A. oryzae* is a naturally occurring phenotype or escaped *A. oryzae* can survive in nature.

All this points to an incomplete knowledge base for *A. oryzae*. However, it is apparent that aflatoxin production is not a concern for established *A. oryzae* strains under usual conditions of cultivation. Hazards posed by mycotoxin production by *A. oryzae* are mitigated by the limitations on exposure as a result of expected use scenarios. While some workers might be exposed, much of that exposure would presumably be via an inhalation route rather than an ingestion. They would be exposed mostly to spores of *A. oryzae* during large-scale fermentation. Since the cultures having the *A. oryzae* phenotype do not produce aflatoxin and are not pathogenic, this exposure should not be significant. Spores that escape the manufacturing site would be unlikely to persist in the environment because of less than optimal conditions for germination and growth. As pointed out in the hazard assessment, *A. oryzae,* lacks many survival features possessed by the related *A. flavus*. Therefore, from the information cited above and using the values in the assessment of exposure, significant environmental exposure relevant to aflatoxin production appears unlikely.

2. Other Toxins

There remains some concern for other mycotoxins produced by koji molds. These toxins are less potent then aflatoxins and their production is tied both to strain specificity and culture conditions. However, they can occur even with current domesticated strains, although there are no reports that their production in industrial fermentations have resulted in adverse effects on human health. The most toxic ones, such as cyclopiazonic acid, seem to be produced by a few strains under special conditions. The less toxic ones, such as kojic acid, may be limited by engineering controls on the fermentation process.

The exposure component of the risk for this concern is similar to that described for aflatoxin. Proper conditions of

cultivation should limit production of these toxins and those limit exposure to workers.

3. Other Issues

Allergenicity seems to be related more to the product of the fermentations than to *A. oryzae per se*. Sensitivity to a-amylase in particular, is a significant concern, but one that exists for all aspergilli producing this enzyme. There is thus no incremental risk specific to the use of these fungi.

4. Summary

Thus, risks for *A. oryzae* seem tied primarily to whether aflatoxins could be produced by production strains, although this is mitigated by controls on exposure. At this juncture, the only means to confirm that this does not occur is to test the resultant product for such toxins.

B. Recommendation

It is recommended for the tiered 5(h)(4) exemption. It is also recommended that comment be sought, first, on the conclusion in section III-A.3 of this document that genetic modification of *A. oryzae* cannot inadvertently produce an aflatoxigenic strain and, second, on whether there is a need to differentiate between strains of *A. oryzae* having long histories of safe use and strains similar in phenotype which are more recent isolates.

VI. REFERENCES

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